Independent mechanisms for acquired salt tolerance versus growth resumption induced by mild ethanol pretreatment in *Saccharomyces cerevisiae.*

Elizabeth A. McDaniel[a,*], Tara N. Stuecker[a], Manasa Veluvolu[a], Audrey P. Gasch[b,c], Jeffrey A. Lewis[a,#]

[a] Department of Biological Sciences, University of Arkansas–Fayetteville, Fayetteville, AR 72701

[b] Laboratory of Genetics, University of Wisconsin–Madison, Madison, WI 53713

[c] Great Lakes Bioenergy Research Center, University of Wisconsin–Madison, Madison, WI 53713

[*] Present Address: Department of Bacteriology, University of Wisconsin–Madison, Madison, WI

[#]Corresponding author:

Email: lewisja@uark.edu

ABSTRACT

All living organisms must recognize and respond to various environmental stresses throughout their lifetime. In natural environments, cells frequently encounter fluctuating concentrations of different stressors that can occur in combination or sequentially. Thus, the ability to anticipate an impending stress is likely ecologically relevant. One possible mechanism for anticipating future stress is acquired stress resistance, where cells pre-exposed to a mild sub-lethal dose of stress gain the ability to survive an otherwise lethal dose of stress. We have been leveraging wild strains of *Saccharomyces cerevisiae* to investigate natural variation in the yeast ethanol stress response and its role in acquired stress resistance. Here, we report that a wild vineyard isolate possesses ethanol-induced cross-protection against severe concentrations of salt. Because this phenotype correlates with ethanol-dependent induction of the *ENA* genes, which encode sodium efflux pumps already associated with salt resistance, we hypothesized that variation in *ENA* expression was responsible for differences in acquired salt tolerance across strains. Surprisingly, we found that the *ENA* genes were completely dispensable for ethanol-induced survival of high salt concentrations in the wild vineyard strain. Instead, the *ENA* genes were necessary for the ability to resume growth on high concentrations of salt following a mild ethanol pretreatment. Surprisingly, this growth acclimation phenotype was also shared by the lab yeast strain despite lack of *ENA* induction under this condition. This study underscores that cross protection can affect both viability and growth through distinct mechanisms, both of which likely confer fitness effects that are ecologically relevant.

53 **IMPORTANCE**

54 Microbes in nature frequently experience “boom or bust” cycles of environmental stress.
55 Thus, microbes that can anticipate the onset of stress would have an advantage. One way
56 microbes anticipate future stress is through acquired stress resistance, where cells exposed to a
57 mild dose of one stress gain the ability to survive an otherwise lethal dose of a subsequent
58 stress. In the budding yeast *Saccharomyces cerevisiae*, certain stressors can cross protect
59 against high salt concentrations, though the mechanisms governing this acquisition of higher
60 stress resistance are not well understood. In this study, we took advantage of wild yeast strains
61 to understand the mechanism underlying ethanol-induced cross protection against high salt
62 concentrations. We found that mild ethanol stress allows cells to resume growth on high salt,
63 which involves a novel role for a well-studied salt transporter. Overall, this discovery highlights
64 how leveraging natural variation can provide new insights into well-studied stress defense
65 mechanisms.
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INTRODUCTION

All organisms experience stress and must respond to environmental perturbations throughout their lifetime. Unlike animals, unicellular organisms generally lack the ability to escape stressful environments. Thus, microbes have evolved sophisticated stress defense strategies such as genome rearrangements, small molecule synthesis, and dynamic gene expression programs to enable stress acclimation (1-3). The model eukaryote *Saccharomyces cerevisiae* responds to diverse stresses by coordinating the expression of condition-specific genes with a large, common gene expression program called the environmental stress response (ESR) (4, 5). The ESR encompasses ~15% of the yeast genome, including ~600 repressed genes that are enriched for processes related to protein synthesis and growth, and the ~300 induced genes that encode diverse functions related to stress defense.

The discovery of a coordinated common response to stress suggested a possible mechanism for the long-observed phenomenon of acquired stress resistance and cross protection, where cells pretreated with a mild dose of stress are better able to survive an otherwise lethal dose of severe stress (6-9). In yeast, defective ESR expression correlates with diminished acquired stress resistance, suggesting that stress-activated gene expression changes may serve to protect against future challenges (10, 11). Beyond yeast, acquired stress resistance has been observed in diverse organisms including bacteria, plants, and humans, and has major implications for food production, agriculture, and human health. For example, mild stress induces higher resistance of the food-borne pathogens *Listeria monocytogenes* and *Salmonella typhimurium* to food preservatives (12, 13). Acquired stress resistance has also been implicated in host survival in the form of bile acid tolerance and antibiotic resistance (14, 15). Acquired thermotolerance and drought resistance in agriculturally significant plants is increasingly important due to climate change (16, 17). In humans, short-term fasting protects healthy cells, but not cancer cells, from the toxic effects of chemotherapy drugs (18, 19).

104 Altogether, understanding how cells are able to acquire further resistance has broad
105 applications ranging from agriculture and biotechnology to human health and disease.
106 We have been leveraging natural variation in the yeast ethanol response to better
107 understand the cellular mechanisms governing acquisition to high stress resistance. While
108 mutagenesis studies in laboratory strains of yeast have identified genes and processes
109 necessary for acquired stress resistance (20-23), there are inherent limitations to using a single
110 strain background (24). In the case of yeast, the S288c laboratory strain historically used for
111 large-scale mutagenesis screening is a genetic and physiological outlier compared to wild yeast
112 strains (25, 26). We have previously noted that S288c has an aberrant gene expression
113 response to ethanol (11, 27), which we hypothesized was responsible for the strain's inability to
114 acquire resistance to any other stresses following pre-treatment with mild ethanol (10, 11). We
115 subsequently found that defective induction of antioxidant defenses in response to mild ethanol
116 was responsible for S288c's inability to acquire further hydrogen peroxide (28).
117 In the present study we found that in contrast to S288c, mild ethanol stress induces
118 cross protection against severe salt concentrations in the wild vineyard strain M22. This
119 phenotype correlated with the induction of the Ena sodium efflux pump system by ethanol in a
120 wild vineyard isolate, which was not induced in the S288c-derived common laboratory strain.
121 Because the ENA system has been previously implicated in salt tolerance (29-31), we
122 hypothesized that variation in *ENA* expression was responsible for phenotypic differences
123 across strains. Surprisingly, we found that the *ENA* genes were completely dispensable for
124 ethanol-induced survival of high salt concentrations in the wild strain. Instead, the *ENA* genes
125 were necessary for a novel growth resumption phenotype that we call "ethanol-induced
126 acclimation," where mild ethanol stress allows cells to eventually resume growth on high
127 concentrations of salt. More surprisingly, our common laboratory strain also exhibited ethanol-
128 dependent acclimation to high salt concentrations, even though *ENA* is not induced by ethanol
129 in this strain background. Overall, this study demonstrates that cross protection can affect both

130 viability and growth through distinct mechanisms, both of which likely confer fitness effects that
131 are ecologically relevant.
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133 **RESULTS**
134 **Ethanol Induces Cross Protection Against Severe Salt in a Wild Vineyard Strain**
135 We previously observed that in our S288c-derived common laboratory strain, mild
136 ethanol pretreatment could not induce acquired resistance to severe ethanol concentrations or
137 cross protect against other stresses (10). In contrast, mild ethanol pretreatment did induce
138 further ethanol resistance in the vast majority of wild yeast strains (11). Additionally, we recently
139 discovered that mild ethanol stress can cross protect against oxidative stress in a wild oak strain
140 (28). Because yeast in nature are likely to experience environmental shifts between high
141 concentrations of sugars and high ethanol concentrations, we hypothesized that the inability of
142 ethanol to cross protect against osmotic stress in S288c may be another aberrant acquired
143 stress resistance phenotype in this strain background. We tested this by examining ethanol-
144 induced cross protection against severe salt in a wild vineyard strain background (M22). Cross
145 protection assays were performed by exposing cells to a mild dose of ethanol (5% v/v) for 1
146 hour, and scoring their survival across a panel of 11 increasingly severe doses of NaCl (see
147 Materials and Methods).
148 We found that ethanol pretreatment weakly cross protected against severe NaCl in
149 S288c (Fig. 1), though cross protection was not completely absent as previously reported (10).
150 In contrast, ethanol strongly improved M22's ability to survive otherwise lethal salt
151 concentrations. Notably, S288c had intrinsically higher basal resistance to NaCl. However, the
152 diminished cross protection phenotype of S288c relative to M22 cannot be explained by the
153 higher baseline resistance, as mild NaCl pretreatment did strongly increase S288c's NaCl
154 resistance (Fig. 2). Moreover, the levels of acquired NaCl resistance following mild NaCl
155 pretreatment were similar for both S288c and M22.

156 **Induction of *ENA1* by Ethanol in a Wild Vineyard Isolate**

157 Because acquired stress resistance relies on stress-activated gene expression changes
158 (10, 11), we hypothesized that the phenotypic differences in cross protection between S288c
159 and M22 may be due to differences in ethanol-responsive gene expression. We analyzed our
160 previous ethanol-responsive transcriptome changes (27), specifically looking for candidate salt
161 resistance genes with higher induction by ethanol treatment in M22 compared to S288c. We
162 noticed that *ENA1* encoding a sodium efflux pump known to be involved in salt resistance (29)
163 showed a 4.7-fold induction by ethanol in M22 versus a 1.4-fold decrease in expression in
164 S288c, placing it within the top 25 genes in terms of magnitude of differential ethanol-responsive
165 expression when comparing M22 and S288c.

166 The Ena P-type ATPase sodium efflux pumps are known to play a critical role in
167 maintaining Na+ ion homeostasis in high salt conditions (30-32). In many yeast strain
168 backgrounds, the *ENA* locus consists of a tandem array of nearly identical genes that can vary
169 in copy number (33). S288c contains three copies (*ENA1, ENA2,* and *ENA5)*, whereas M22
170 appears to contain a single copy (34). This single copy is somewhat unusual, in that M22
171 appears to contain a large 3885-bp deletion in *ENA1* relative to that of S288c, which results in a
172 full-length in-frame fusion of *ENA1* and *ENA2* (34).

173 *ENA* copy number has been linked to high salt tolerance (25–27), likely explaining why
174 S288c has higher basal NaCl tolerance than M22. In S288c, the *ENA* genes are lowly
175 expressed under standard growth conditions (24), but are highly induced in response to saline
176 or alkaline pH stresses (23). Including our previous studies, there are currently no reports of
177 *ENA* induction by ethanol stress in the S288c, suggesting that this mode of *ENA* regulation may
178 have been lost in the laboratory strain background (see Discussion).

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181 **The ENA System is not Required for Ethanol-Induced Survival in Severe Salt, but is**
182 **Required for Growth Resumption During Salt Acclimation**

183 Because variation in *ENA* expression is linked to basal salt resistance, we hypothesized
184 *ENA1* induction by ethanol may protect M22 from otherwise lethal salt stress. To test this, we
185 deleted the entire *ENA* region in M22 and examined its role in ethanol-induced acquired NaCl
186 resistance. Surprisingly, we found no defect in acquired salt resistance in the M22 *ena*Δ mutant
187 (Fig. 3A). One possible explanation is that the ENA system is not necessary for short-term
188 exposure to acute NaCl, but is instead required for long-term survival. Thus, we measured
189 acquired NaCl resistance in the M22 *ena*Δ strain over 24 hours. Even after 24 hours, the M22
190 *ena*Δ strain acquired NaCl resistance equivalently to the wild-type strain, and had at most a mild
191 basal NaCl resistance defect (Fig. 3A). We did notice that for ethanol pre-treated wild-type cells,
192 the plating density significantly increased over time (Fig. 3B). This suggested that the ethanol
193 pretreatment enabled cells to acclimate and resume growth on high concentrations of NaCl, and
194 that this acclimation phenotype may be Ena dependent.

195 To further examine the role of the Ena in acclimation to high salt stress after ethanol
196 pretreatment, we measured growth in liquid media, with or without mild ethanol pretreatment
197 (Fig. 4A). Ethanol pretreatment allowed wild-type M22 cells to acclimate and resume growth in
198 1.25M NaCl, and this growth resumption was completely abolished in the M22 *ena*Δ mutant.
199 These data suggest that two cross protection phenotypes—survival vs. growth—have distinct
200 cellular mechanisms.

201 In light of the distinct requirement of *ENA* for growth resumption on salt but not survival
202 in the M22 background, we examined whether S288c was able to acclimate and resume growth
203 on high salt following ethanol pretreatment. Indeed, ethanol pretreatment induced growth
204 resumption in S288c (Fig. 4B), which was abolished in the S288c *ena*Δ mutant. This was
205 somewhat surprising, considering that *ENA1* is not induced by ethanol in S288c under these
206 conditions (see Discussion).

207 **DISCUSSION**

208 In this study, we initially sought to understand how ethanol cross protects against severe
209 salt stress. We found that ethanol-induced cross protection against severe salt was weaker in
210 the common lab strain S288c when compared to the wild vineyard strain M22. We examined
211 previous transcriptional profiling of the yeast ethanol response to identify candidate genes
212 induced by ethanol in M22 but not S288c (27). Based on these data, we identified the ENA
213 system as a prime candidate to test. The ENA system uses the hydrolysis of ATP through P-
214 type ATPases to transport sodium out of the cell against the electrochemical gradient (22), and
215 mutants lacking *ENA* function are salt sensitive (29-31). Interestingly, the *ENA* locus of many
216 yeast strains including S288c and M22 appears to be the result of a recent introgression from *S.*
217 *paradoxus* and shows significant copy number variation across strains (33). Other strains have
218 a single non-S288c-like *ENA6* gene that does not share sequence similarity to the *ENA* genes
219 from *S. paradoxus* (26, 30). Genetic mapping studies have linked both copy number variation
220 and polymorphisms in the *ENA* region to variation in NaCl and LiCl tolerance (26, 35-37).

221 Thus, we were somewhat surprised to find that the ENA system of M22 was completely
222 dispensable for survival in high salt following ethanol pretreatment. Instead, the ENA system
223 was required for a novel ethanol-induced cross-protection phenotype that allows for acclimation
224 and subsequent growth resumption in the presence of high salt. Notably, the vast majority of
225 studies examining salt sensitivity phenotypes for *ENA* have been performed by growing cells on
226 salt-containing plates, which cannot easily distinguish between viability and growth. Both
227 phenotypes are likely important in natural environments. Wild yeast cells growing on fruit such
228 as the M22 vineyard strain may experience simultaneous or fluctuating hyperosmotic stress and
229 ethanol stress, which could explain the evolution of cross protection.

230 Because we were able to separately examine survival and growth, we reassessed the
231 role of the ENA system in S288c. Surprisingly, we found that while ethanol only weakly induced
232 higher survival on high salt in the S288c background, ethanol-induced acclimation to high salt

233 was similar between the two strains. This ethanol-induced acclimation phenotype in S288c was
234 also *ENA* dependent, despite the lack of induction of *ENA* by ethanol in this strain. Notably,
235 *ENA* is known to be induced by NaCl in the S288c background (38), which is likely necessary
236 for growth resumption on high salt. Additionally, basal *ENA* expression is higher in S288c
237 compared to M22 (11, 27), likely due to copy number variation (our S288c-derived laboratory
238 strain contains three *ENA* copies, while M22 contains a single copy (34)). It is likely that other
239 ethanol-induced genes and processes are necessary for ethanol-induced acclimation to high
240 salt concentrations.

241 The striking induction of the ENA system by ethanol in M22 but not S288c implies
242 regulatory differences between the two strains. Recently, natural variation in the promoter
243 region of *ENA6* in a sake strain was shown to increase Ena6p expression and thus increase salt
244 tolerance (37). In this strain, a 33-bp deletion in the promoter eliminates glucose repression by
245 eliminating repressor binding sites for the Nrg1p and Mig1/2p transcription factors. In contrast,
246 we hypothesize that the novel regulation of *ENA1* by ethanol in M22 is likely not due to promoter
247 variation. Comparing the promoters of *ENA1* between the S288c and M22 backgrounds reveals
248 two SNPs and a 20-bp AT repeat insertion within a 20-bp poly-AT repeat region. However,
249 these promoter differences do not alter or introduce any predicted transcription factor binding
250 sites, suggesting promoter variation is unlikely responsible for the observed expression
251 differences between the two strains. Instead, it is likely that *trans* regulatory variation is
252 responsible for the novel induction by ethanol in the M22 background. The phenotypic
253 consequences of this novel induction of *ENA1* by ethanol in the M22 strain remain an
254 unresolved question, as S288c exhibits a similar growth resumption phenotype. Nonetheless,
255 these findings expand our knowledge of the ENA system's role in stress defense mechanisms,
256 and highlight the power of using natural variation to yield new insight into even previously well-
257 studied aspects of cellular physiology, such as the ENA system.
258

259 **MATERIALS AND METHODS**

260 **Strains and Growth Conditions**

261 Strains and primers used in this study are listed in Tables 1 and 2, respectively. The
262 entire *ENA* region was deleted by homologous recombination and replacement with a KanMX4
263 drug resistance marker in the haploid MATa strain BY4741. This strain was used as a genomic
264 template for introducing the *ena1-5*Δ::KanMX4 allele into different strain backgrounds. To
265 generate homozygous *ena*Δ diploids in the S288c strain background, the *ena1-5*Δ::KanMX4
266 region was amplified and transformed into MATa and MATα haploid derivatives of DBY8268,
267 which were then mated together. To generate homozygous *ena*Δ diploids in the M22
268 background, the *ena1-5*Δ::KanMX4 region was transformed into the diploid M22 strain, resulting
269 in an *ena*Δ heterozygote. M22 is capable of mating-type switching, and thus sporulation and
270 dissection yielded homozygous *ena*Δ diploids. Homozygous deletions were verified by
271 diagnostic PCR.

272 All strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C with
273 orbital shaking (270 rpm). Optical density was recorded using a Unico spectrophotometer.
274 Sporulation was achieved by growing cells to saturation for 2 days in YPD, harvesting by
275 centrifugation, resuspending in 1% potassium acetate, and incubating for up 3-5 days at 25°C
276 with shaking.

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278 **Acquired Stress Resistance Assays**

279 The acquired stress resistance assays were performed as described in (28). Briefly, cells
280 were grown to overnight to saturation, sub-cultured into in 30-mL fresh media, and then grown
281 for at least 8 generations into exponential phase (OD_{600} of 0.3-0.6) to reset any cellular memory
282 of starvation stress (39). Each culture was split and pretreated with either a mild “primary” stress
283 or a mock (equivalent concentration of water) control. Primary stresses included either 5% v/v
284 ethanol or 0.4 M NaCl. Cells were incubated with the pretreatment for 1 hour and then collected

285 by mild centrifugation at 1,500 *x g* for 3 min to remove the primary stress. Cells were
286 resuspended in fresh media to an OD_{600} of 0.6, and then diluted 3-fold into a microtiter plate
287 containing a panel of severe NaCl doses ranging from 1.2 M to 3.2 M (0.2 M increments).
288 Plates were sealed breathable Rayon films (VWR), and incubated with secondary stress at
289 30ºC with 800 rpm shaking in a VWR Symphony Incubating Microplate Shaker. Secondary
290 treatments were for 2h unless otherwise noted. Following secondary treatment, 4 µl of a 50-fold
291 cell dilution was spotted directly onto YPD agar plates and grown for 48 hours at 30°C. Viability
292 at each dose was scored using a 4-point semi-quantitative scale that compared survival in each
293 secondary dose against an unstressed (YPD only) control: 100% viability = 3 points, 50-90%
294 viability = 2 points, 10-50% viability = 1 point, and 0% viability = 0 points. An overall tolerance
295 score was calculated as the sum of scores across all 11 stress doses. Acquired stress
296 resistance assays were performed in biological triplicate, and raw phenotypic data can be found
297 in Table S3. A detailed acquired stress resistance assay protocol can be found on protocols.io
298 under doi dx.doi.org/10.17504/protocols.io.g7sbzne. Statistical analyses were performed using
299 Prism 7 (GraphPad Software).
300
301 **Ethanol-Induced Growth Resumption Analysis**
302 To assess ethanol-induced growth resumption in the presence of salt, cells were given
303 mild primary ethanol (5% v/v) or mock pretreatments as described for the acquired resistance
304 assays. Following 1 hour pretreatment, cells were gently centrifuged at room temperature for 3
305 minutes at 1500 *x g*, and then resuspended in YPD containing 1.25 M NaCl at an OD_{600} of 0.1.
306 Five ml of each sample was transferred to a glass test tube and incubated at 30ºC at 270 rpm.
307 The OD_{600} of all samples was then manually measured with a Unico spectrophotometer over
308 approximately 72 hours. Growth assays were performed in biological triplicate.
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466 **TABLES**

467 **Table 1. Strains used in this study.**

Strain	Background	Genotype	Source
DBY8268	S288c (lab strain)	*MATa/MATα ura3-52/ura3Δ ho/ho GAL2/GAL2*	David Botstein
JL187	BY4741	*MATa his3Δ leu2Δ met15Δ ura3Δ ena1-5Δ::kanMX*	This study
JL505	DBY8268	*MATa ho GAL2 ura3-52 or ura3Δ*	
JL506	DBY8268	*MATα ho GAL2 ura3-52 or ura3Δ*	
JL1127	DBY8268	*MATa ho GAL2 ura3-52 or ura3Δ ena1-5Δ::KanMX*	This study
JL1128	DBY8268	*MATα ho GAL2 ura3-52 or ura3Δ ena1-5Δ::KanMX*	This study
JL1131	DBY8268	*MATa/MATα ho/ho GAL2/GAL2 ura3-52/ura3Δ ena1-5Δ::KanMX/ena1-5Δ::KanMX*	This study
M22	wild vineyard strain	*MATa/MATα*	Robert Mortimer
JL213	M22	*MATa/MATα ena1-5Δ::KanMX/ena1-5Δ::KanMX*	This study
JL214	M22	*MATa/MATα ena1-5Δ::KanMX/ENA1-5*	This study

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469 **Table 2. Primers used in this study.**

Primer Name	Sequence	Notes
ENA1_KanMX_up_484	CATTTATTTCCTACTTCTATGACGTTTGTTAGGGCAGGGATGTAGTACGCTGCAGGTCGAC	Contains 45-bp homology to a region 448-bp upstream of the *ENA1* start codon. Paired with "ENA5_KanMX_down_92" to amplify the KanMX4 for generating *ena1-5*ΔKanMX4.
ENA5_KanMX_down_92	CTCATTACCTAAATTTGTTTATGTTCGGTAGCCCTAAAGGAGCTTCATCGATGAATTCGAGCTCG	Contains 45-bp homology to a region 92-bp downstream of the *ENA5* stop codon. Paired with "ENA5_KanMX_up_448" to amplify the KanMX4 for generating *ena1-5*ΔKanMX4.
kanB	CTGCAGCGAGGAGCCGTAAT	Common MX4 cassette primer from (40). Paired with "ENA 1kb up F" to verify insertion of KanMX4 into the *ENA* locus.
ENA 1kb up F	GTCAATATTTTAGGGTTATCGGT	Anneals 1-kb upstream of the *ENA1* start codon in S288c. Paired with "ENA 1kb down R" to amplify *ena1-5*ΔKanMX (3 kb product).
ENA 1kb down F	GTCAATATTTTAGGGTTATCGGT	Anneals 1-kb downstream of the *ENA5* stop codon in S288c. Paired with "ENA 1kb up F" to amplify ena1-5ΔKanMX (3 kb product).
ENA1_qPCR_up	ACACTGACAGCCCAGTCAAGGAAT	Anneals 2,921-bp downstream of the *ENA1* start codon. Paired with "ENA1_qPCR_down" to amplify 181-bp product. Used to verify loss of *ENA1* during *ena1-5*ΔKanMX mutant construction.
ENA1_qPCR_down	ATTGTGAATGCAATGGCGAGACCC	Anneals 3,198-bp downstream of ENA1 start codon. Paired with "ENA1_qPCR_up" to amplify 181-bp product. Used to verify loss of *ENA1* during *ena1-5*ΔKanMX mutant construction.

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471 **FIGURES**

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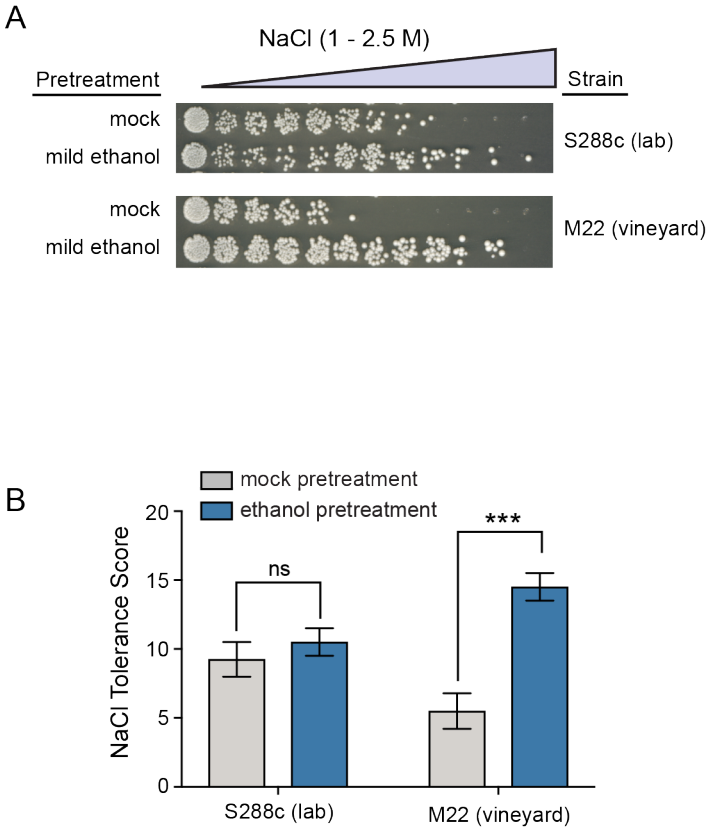

485 **Fig. 1. Ethanol induces cross protection against severe salt in a wild vineyard isolate. A)**
486 A representative NaCl cross protection assay. S288c (DBY8268 lab strain) and M22 (wild
487 vineyard strain) were exposed to mild 5% ethanol or a mock (5% water) pretreatment for 1 hour,
488 washed, and then exposed to 11 increasingly severe doses of NaCl for 2 hours before plating to
489 score viability. **B)** NaCl tolerance scores were calculated from the viability across each of the 11
490 doses (see Methods). Error bars denote the standard deviation of four independent biological
491 replicates. Asterisks indicate significantly higher resistance in ethanol-pretreated versus mock-
492 pretreated cells (*** $P < 0.001$, ns = not significant ($P > 0.05$), *t*-test).

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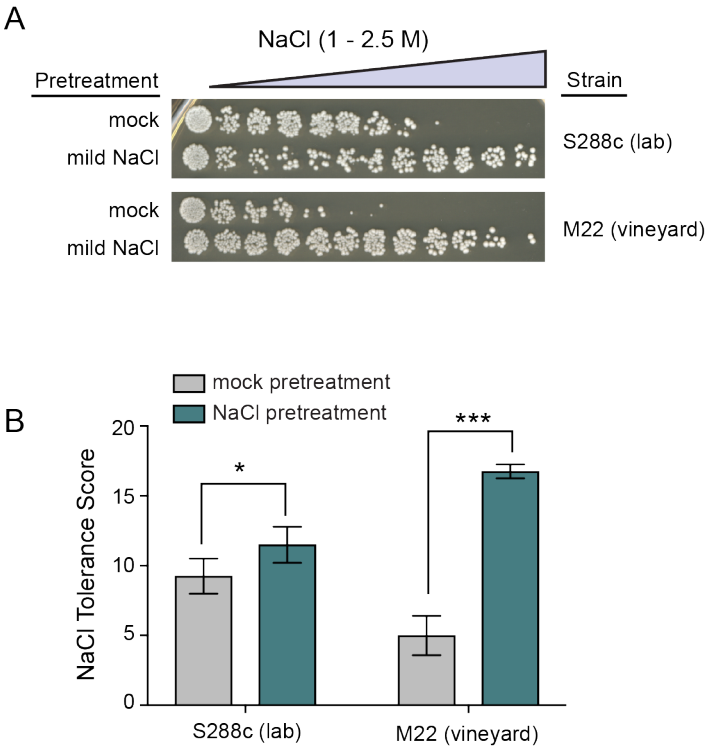

Fig. 2. NaCl allows acquisition of even higher NaCl resistance in both the lab and vineyard strains. A) A representative acquired NaCl resistance assay is shown for both S288c (DBY8268 lab strain) and M22 (wild vineyard strain). Cells were split and exposed to either 0.4 M NaCl or a mock (media only) pretreatment for 1 hour, washed, exposed to 11 doses of severe NaCl for 2 hours, and then plated to score viability. **B)** Salt tolerance scores across each of the 11 doses are plotted as the mean and standard deviation of three independent biological replicates. Asterisks indicate significantly higher resistance in NaCl-pretreated versus mock-pretreated cells (* $P < 0.05$, *** $P < 0.001$, *t*-test).

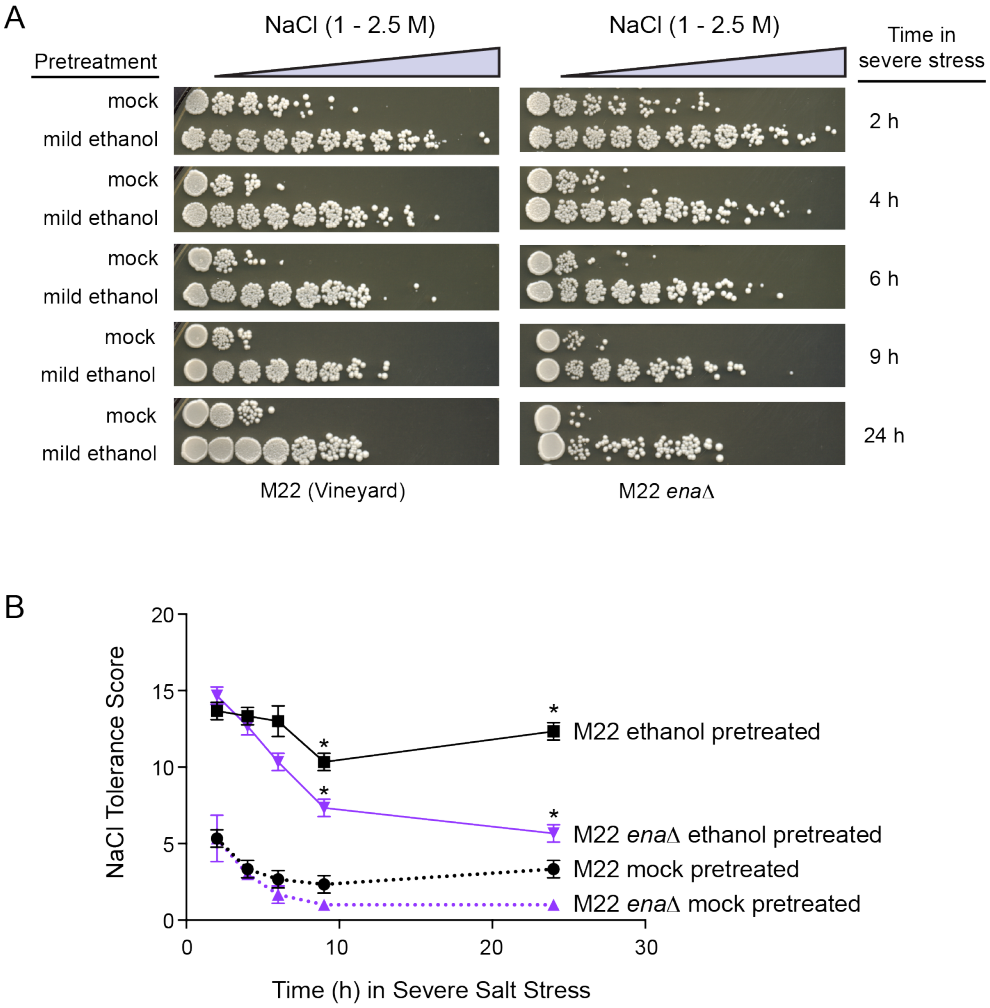

Fig. 3. The ENA system is not necessary for ethanol-induced survival against severe salt. **A)** Representative NaCl cross protection assays are shown with increasing amounts of time in the severe secondary stress doses for Wild Type M22 and the M22 *ena*Δ mutant. **B)** Salt tolerance scores across each of the 11 doses were calculated for each of the timepoints from Panel A. Error bars denote the standard deviation of three independent biological replicates. Asterisks indicate timepoints with significantly higher resistance in M22 compared to the *ena*Δ mutant (*$P < 0.01$, *t*-test).

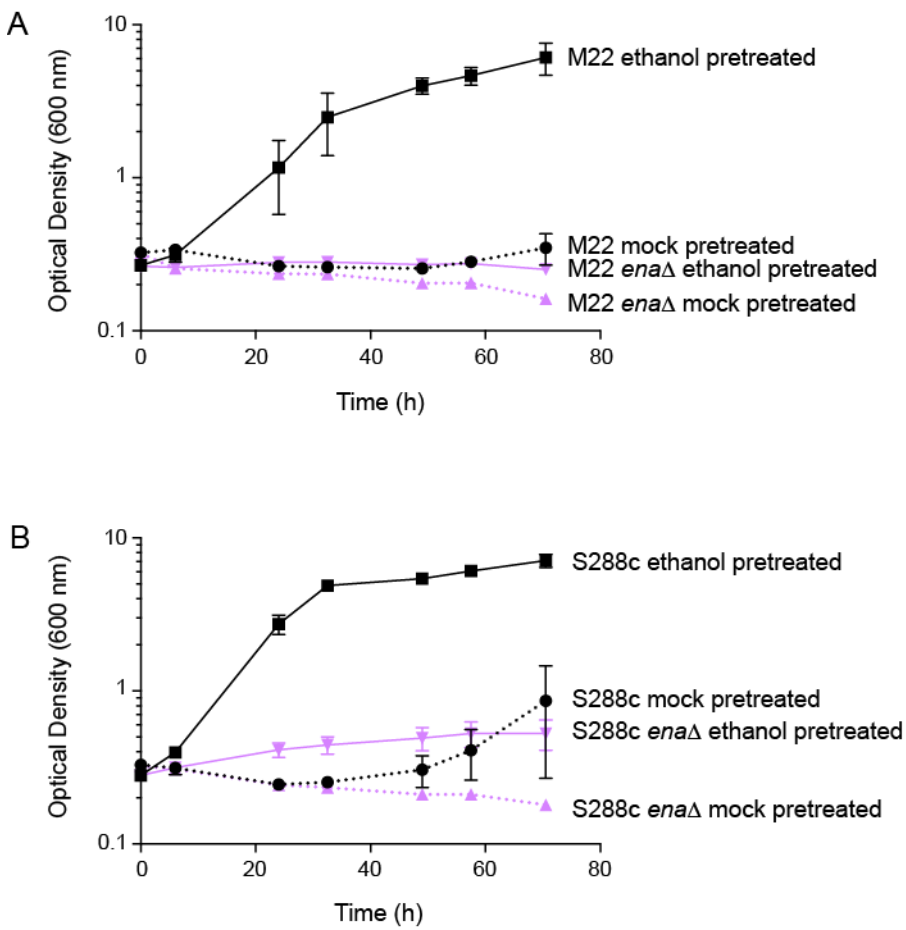

Fig. 4. The ENA system is required for ethanol-induced growth resumption in severe salt in both S288c and M22. A) M22 and M22 *ena*Δ *(*JL213) and **B)** S288c (JL505) and S288c *ena*Δ (JL1131) were exposed to mild 5% ethanol or a mock (5% water) pretreatment for 1 hour, washed, and then resuspended in YPD containing 1.2 M NaCl. Growth was then measured over the course of 3 days. Error bars denote the standard deviation of three independent biological replicates.